Prostate Cancer Expression of Runt-Domain Transcription Factor Runx2, a Key Regulator of Osteoblast Differentiation and Function

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BACKGROUND. Prostate cancer (CaP) bone metastases express numerous proteins associated with bone cells. Specific transcription factors, including Runx2, regulate the expression of many bone-related factors in osteoblasts. Expression of these transcription factors in CaP may be linked to the ability of CaP bone metastases to influence bone remodeling.
METHODS. CaP tissues and cell lines were analyzed for expression of Runx2 mRNA by RT-PCR and in situ hybridization, and protein by immunohistochemistry, Western blotting, and electrophoretic mobility shift assays (EMSA).
RESULTS. Runx2 mRNA and protein were detected in CaP tissues and cell lines. A specific Runx2: OSE2 complex could be formed with PC-3 nuclear extracts.
CONCLUSIONS. Expression of Runx2 in CaP may be the molecular switch that is associated with expression of various bone-specific factors in CaP. In turn, expression of these factors can influence bone remodeling and possibly play a role in the growth and survival of CaP in bone.
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KEY WORDS: **prostate cancer; bone metastases; Cbfa1; OSE2; BMP-2**

INTRODUCTION

Prostate cancer (CaP) is the most commonly diagnosed malignancy in men and is often associated with bone metastases. CaP bone lesions can be lytic or sclerotic, with the latter predominating. The molecular mechanisms by which CaP alters the balance of normal bone metabolism are poorly understood but it has been hypothesized by Koeneman et al. [1] that CaP cells are osteomimetic in their ability to thrive and grow in the bone milieu. CaP expresses proteins associated with osteoblasts such as osteocalcin [2], receptor activator of NFkB ligand (RANKL) [3], bone sialoproteins [4], osteopontin [5], steoprotegerin (OPG) [3], and bone morphogenetic proteins (BMPs) [6,7]. Expression of several of these proteins is further upregulated in metastatic bone lesions when compared to primary CaP. Increased transcriptional activation of bone-related factors in CaP cells is a candidate mechanism for involvement in the deregulation of bone remodeling associated with CaP bone metastases.

Runt-domain transcription factor Runx2 (also called Pebp2αA, Cbfa1, AML-3, or Osf2) is a transcription factor essential for osteoblast differentiation [8,9]. Runx2 regulates transcription of many bone-related factors in osteoblasts, including osteocalcin [8], bone sialoproteins [10], osteopontin [11], collagen type I [12], and OPG [13] through an osteoblast-specific *cis*-acting element termed OSE2. Targeted disruption of *runx2* results in complete lack of bone formation [14,15], while mutations are responsible for the genetic disease cleidocranial dysplasia [16,17]. *Runx2* gene expression is upregulated early in development in association with mesenchymal condensations and at late stages during bone mineralization [8,18]. Factors involved in osteoblast differentiation, such as the BMPs [19] and

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1,25-$(OH)_2$-vitamin D_3 [20], have been reported to regulate Runx2 expression.

Three isoforms of the *Runx2* gene have been identified by differential promoter usage and have varying N-terminal sequences (Fig. 1A). Isoform I, with N-terminal amino acids MRIPV, was cloned from *ras*-transformed fibroblasts and has been also detected in spleen, thymus, and T-cell lines [21,22]. Recently, isoform I has been observed in mesenchymal tissues, preosteoblasts, and chrondrocytes [19,23]. Isoform II, with the N-terminal sequence MASNS, was revealed as the (silent) gene activated by retroviral integration at the *til*-1 locus in virus accelerated lymphomas of CD2-*myc* transgenic mice [24]. This isoform was detected in long bone osteoblasts and osteosarcoma cell lines, while T-cell and fibroblast cell lines were negative.

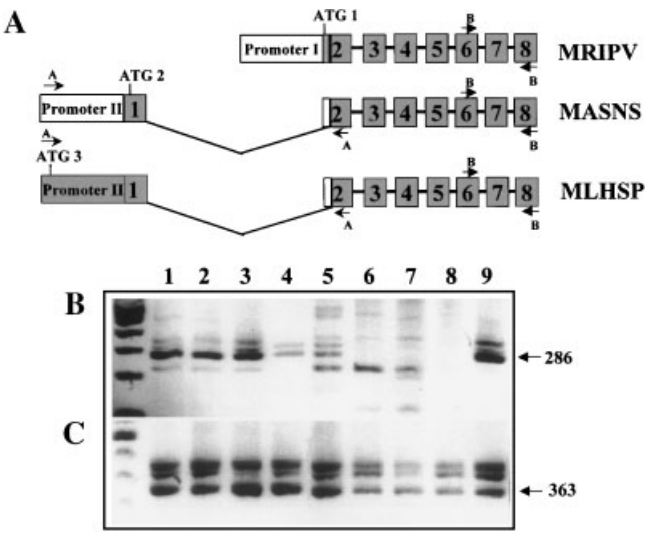

Fig. 1. Runx2 isoforms and their detection in CaP cell lines by RT-PCR. **A**: Diagram of the structural differences between the three Runx2 isoforms. There are three isoforms of *runx2* starting from alternative ATG sites. Isoform I begins at a start codon in exon 2 and results in a protein starting with MRIPV sequence. Isoform II uses a start codon in exon I and codes for a protein with the N-terminal sequence MASNS. Isoform III starts at an ATG located 5′ from exon I, which is allocated as the promoter region for isoform II [25,26]. The N-terminal sequence of isoform III is MLHSP. Grey boxes represent exons and white boxes represent promoter regions. For isoform III, promoter II is represented by a grey box since it is part of the coding region. The primers used to amplify *runx2* messages are illustrated by arrows and are labeled either set A or B. **B**,**C**: RT-PCR of analysis of *runx2* expression in CaP cell lines. For B and C, lanes are as follows. **Lanes 1**: DU 145, **2**: PC-3, **3**: B-PC-3, **4**: N-PC-3, **5**: LNCaP, **6**: C4, 7: C4-2, **8**: C4-2B, and **9**: MG-63. B: Using primer set A, which amplifies a 286 bp region at the 5′-end of *runx2*, multiple bands were amplified, with two main bands of ~286 and ~320 bp detected in DU 145, PC-3, B-PC-3, N-PC-3, LNCaP, and the positive control, MG-63. C: Primer set B, which amplifies a 363 bp region at the 3′-end of *runx2* amplified three band of ~363, ~425, and ~440 bp in all CaP cell lines

Isoform III was isolated from a mouse osteoblast cDNA library and has an N-terminus beginning with the amino acid sequence MLHSP [8]. This isoform has been detected only in bone and osteoblasts. Several reports define the type III translation start site as part of the 5′-untranslated region of the type II isoform [25,26]. Interestingly, Harada et al. [27] reported that all three isoforms upregulated expression of osteocalcin, osteopontin, and type I collagen when stably transfected into mouse embryonic fibroblast C3H10T1/2 cells, with isoform II stimulating the highest osteocalcin expression. They also observed that the type I and II isoforms upregulated alkaline phosphatase, suggesting a role for these two isoforms in early differentiation of osteoblasts. Banerjee et al. [19] reported that isoforms I and II increased reporter activity from an osteocalcin promoter in the osteoblastic cell line ROS 17/2.8, and observed increased levels of isoform II with osteoblast differentiation. Unfortunately, these studies did not examine isoform III activity or expression.

There have been two recent reports on expression of Runx2 in CaP cell lines. Yeung et al. [28] reported that PC-3, an androgen-independent CaP cell line isolated from a bone metastasis, expresses high levels of osteocalcin through transcriptional regulation by Runx2, Jun-D/Fra-2, and Sp-1. Lin et al. [29] demonstrated that C4-2B, a LNCaP subline, which was also isolated from a bone metastasis, expresses Runx2 protein and mRNA. In this study, we report the detection of Runx2 mRNA and protein in tissue samples representing various stages of CaP progression, and in CaP cell lines. Functional analyses demonstrated interactions between Runx2 and OSE2 DNA-binding elements found in the promoters of bone specific genes. We hypothesize that Runx2 expressed by CaP may contribute to the upregulation of various bone-related factors in CaP cells and their ability to disrupt the balance of bone remodeling when present in bone metastases.

MATERIALS AND METHODS

Cell Culture

Tissue culture media and supplements were obtained from Gibco Life Technologies, Inc. (Rockville, MD). All CaP cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) under standard culture conditions. The CaP cell lines used in this study were DU 145 and PC-3 (ATCC, Rockville, MD) and the PC-3 sublines, N-PC-3 (noninvasive) and B-PC-3 (invasive) (gifts from Dr. M Stearns, Medical College of Pennsylvania, Philadelphia, PA); and LNCaP (ATCC) and its sublines, C4, C4-2, and C4-2B (Urocore, Oklahoma City, OK). Human osteosarcoma cell lines MG-63 and Saos-2, and the pre-myoblast line C_2C_{12} were obtained from

the ATCC. MG-63 and Saos-2 were cultured in DME medium supplemented with 10% FBS under standard culture conditions. C_2C_{12} cells were cultured in RPMI 1640 medium supplemented with 10% FBS. For BMP-2 studies, PC-3, LNCaP, and C_2C_{12} grown to 90% confluency were switched to phenol red-free RPMI 1640 medium supplemented with 2% FBS for 24 hr, then treated with BMP-2 (200 ng/ml, Genetics Institute, Cambridge, MA) in phenol red-free RPMI 1640 medium for 0, 3, 6, 12, and 24 hr. The cells were then scraped and pelleted for isolation of nuclear extracts (see below: Preparation of Nuclear Extracts).

Tissue Samples

For in situ hybridization (ISH) and immunohistochemistry (IHC) studies, human prostate tissue samples were obtained from radical prostatectomies ($n = 16$) or rapid autopsies ($n = 12$). Tissues were fixed in 10% buffered formalin and embedded in paraffin. Bone samples were fixed in formalin and decalcified in 5% formic acid prior to embedding. Five-micrometer sections were used for IHC and ISH. Slides were baked at 60°C overnight prior to deparaffinization in xylene (three times steps) and rehydration in a series of 100%, 95%, and 70% ethanol (EtOH) rinses. Tissue integrity was assessed by hematoxylin and eosin staining, and the presence of CaP cells was assessed by PSA staining. For RT-PCR studies, samples of benign prostate and areas containing at least 80% cancer were microdissected from radical prostatectomy specimens. Eleven samples from localized disease with a median Gleason score of 7 (range 6–9), a mean PSA of 15.36 ± 9.63 ng/ml (range 2.51–111.5), and stages ranging from T2A-T3CN+ were used.

RT-PCR

Total RNA was isolated from cell lines or prostate tissues using STAT 60 (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. RT-PCR was performed as previously described [30]. Briefly, cDNA was generated from 5 μg total RNA using SuperScript II (Gibco) with random hexamers. Hot-start two-step PCR was performed: 1 cycle at 80°C for 3 min; 25–35 cycles of 94°C for 10 sec, 69°C for 1 min; and a final extension period at 72°C for 7 min. Two sets of primers were used to amplify fragments of human *runx2*. Primer set A was designed to amplify a 286-bp fragment at the 5′-end of the gene (Accession # AF010284, 1-286): 5′ (ATGCTTCATTCGCCTCACAAACAAC) and 3′ (TGAAGCGCCGGCTGGTGCTC). Primer set B was designed to amplify a 363-bp fragment at the 3′-end of *runx2* (Accession # L40992, 698–1061): 5′ (AGTCTTCCCCGCCGTGGTCCTATGAC) and 3′ (GGAGTGCTGCTGGTCTGGAAGGG).

In Situ Hybridization (ISH)

The 286-bp amplicon of *runx2* generated with primer set A was cloned into the pGEM-T vector (Promega, Madison, WI). DNA sequencing was used to confirm the identity and determine the orientation of the insert. Purified plasmid was linearized using either *Nco*I or *Not*I and digoxigenin-labeled anti-sense and sense riboprobes were generated using a T7 and SP6 in vitro transcription kit (Roche, Indianapolis, IN). The probes were separated on a 1.2% agarose gel and relative amounts of RNA were quantified using a digital image analyzer (Alpha Innotech Corp., San Leandro, CA). ISH was performed using the Ventana gen^{II} automated ISH system (Ventana Medical Systems, Inc., Tuscon, AZ). Hybridization was performed using 25 ng/slide sense or anti-sense riboprobes at 50°C for 5 hr in hybridization buffer (8 mM Tris, pH 8.0, 50% deionized formamide, 10% dextran sulfate, 1× Denhardt's solution, 0.3 M NaCl, 0.8 mM EDTA, 2 mg/ml Yeast tRNA, 10.0 mM DTT), with subsequent rinses of 2×, 1×, 1× SSC buffer at 48°C. Bound probes were detected with anti-digoxigenin monoclonal antibody (1:2,000, Sigma, St. Louis, MO) in combination with biotinylated rabbit anti-mouse antibodies, streptavidin-HRP, and DAB. Sections were counterstained with Harris' hematoxylin prior to mounting.

Immunohistochemistry (IHC)

Antigen retrieval was performed using 10 mM citrate buffer, pH 6, in a pressure cooker for 10 min. Endogenous peroxidase was quenched by incubation of tissues in 0.3% hydrogen peroxide in PBS for 10 min. Non-specific binding was blocked with serum block (5% goat, 5% horse, 5% chicken sera) in PBS for 1 hr at room temperature. Endogenous biotin was blocked using an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA). Anti-Osf-2 rabbit serum (1:250, gift from Dr. G. Karsenty, Baylor University, Houston, TX [31]) was applied to the sections which were incubated overnight at 4°C in a humidified chamber. Negative control sections were incubated with rabbit serum under the same conditions. To detect immunoreactivity, sections were incubated with biotin-conjugated goat anti-rabbit antibody for 30 min at room temperature and processed with an ABC kit (Vector Laboratories, Burligame, CA) and DAB as the substrate. Sections were counterstained in Harris' hematoxylin and blued in ammonia water prior to mounting.

Preparation of Nuclear Extracts

Nuclear extracts were prepared from 90% confluent PC-3, LNCaP, C_2C_{12} and MG-63 cells, and Saos-2 cells which were cultured for 16 days in the presence of

50 μg/ml ascorbic acid (Sigma, St. Louis, MO) and 10 mM β-glycerophosphate (Sigma). Nuclear extracts were isolated by standard methods [32] with the following modifications. Approximately 30×10^6 cells were scraped in 10 ml Hank's buffered saline (Gibco Life Technologies) and centrifuged for 3 min at 500*g*. The cell pellet was rinsed with five times the pellet volume (PV) of hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) and centrifuged at 500*g* for 1 min. The pellet was resuspended in 3PV of hypotonic buffer with Complete™ protease inhibitors (Roche, Indianapolis, IN), vortexed and placed on ice for 30 min. The lysate was spun at 3,300*g* for 20 min at 4°C and the supernatant was aliquoted and stored at −80°C as the cytosolic fraction. The pellet (nuclei) was resuspended in ½PV of low salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.02 M KCL, 0.2 mM EDTA, 0.5 mM DTT and Complete™ protease inhibitors) to which ½PV of high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 1.0 M KCL, 0.2 mM EDTA, 0.5 mM DTT, Complete™ protease inhibitors) was added drop wise while vortexing, to prevent local concentrations of high salts. The nuclear extract was placed on ice for 30 min, then centrifuged at 16,000*g* for 30 min at 4°C. The supernatant was aliquoted and stored at −80°C until use. Protein levels were determined using the Bio-Rad D_C Protein Assay (BioRad, Hercules, CA).

Western Blotting

Nuclear extract and cytosolic fractions (35 μg per lane) were resolved by 12.5% SDS–PAGE and transferred to PVDF membranes (BioRad, Hercules, CA) under standard conditions. After blocking with NAP-*Sure*Blocker (Geno Technology, Inc., St. Louis, MO) for 2 hr, Runx2 protein was detected using anti-Osf-2 rabbit serum (1:1,000, gift from G. Karsenty, against amino acids (AAs) 16-31, SFFWDPSTSRRFSPPS), rabbit anti-AML-3 polyclonal antibody (1 μg/ml, Calbiochem, San Diego, CA, against AAs 333-348 (TDVPRRISDDDTATSD) of Accession # Q13950) or rabbit anti-Pebp2αA polyclonal antibody (1 μg/ml, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, against AAs 294-363 (PQVATYHRAIKVTVDGPREPRRHRQKLDDSKPSLFSDRLSDLGRIPHPSMRVGVPPQNPRPSLNSAPSPFOF) of mouse Pebp2αA). Membranes were incubated with primary antibodies for 2 hr at room temperature and after extensive rinses in Tris buffered saline (TBS) plus 0.1% Tween 20, blots were incubated with goat anti-rabbit HRP-conjugated secondary antibody (1:3,000, Amersham, Piscataway, NJ) for 1 hr. Amersham ECL was used for detection with Kodak X-OMAT AR film.

Electrophoretic Mobility Shift Assay (EMSAs)

EMSAs were performed using conditions similar to those previously reported [18,22]. The oligonucleotide sequences used as probes or competitors were AML binding site consensus sequence 5′-CGTATTAACCACAATACTCG-3′ [18] and human osteocalcin OSE2 5′-CGCAGCTCCCAACCACATATCC-3′. Double-stranded DNA were prepared by annealing after heating to 95°C and end-labeled with [γ-^{32}P]ATP (Amersham) using T4 polynucleotide kinase (Promega, Madison, WI). Nuclear extracts from LNCaP and PC-3 (20 μg) were incubated with 50 fmol of dsDNA probes for 30 min at 37°C in a buffer containing: 20 mM Tris, pH 8, 10 mM NaCl, 3 mM EDTA, 0.05% Nonidet P-40, 2 mM DTT, 4% glycerol, 1 mM $MgCl_2$, 1 μg poly dI-dC (Amersham). The samples were subjected to electrophoresis on a 4% non-denaturing polyacrylamide gel in 0.5× TBE at 130 V for 6 hr. For supershift experiments, 2 μg of either anti-AML-3 or anti-Pebp2αA polyclonal antibodies or rabbit IgG (Vector Laboratories, Burlingame, CA) was added to the reactions 10 min prior to gel electrophoresis. In competition experiments, unlabeled DNA probe (100×) was incubated with the nuclear extracts for 10 min prior to addition of the labeled probe.

RESULTS

Runx2 mRNA

Runx2 message was detected in DU 145, PC-3, B-PC-3, N-PC-3, and LNCaP (Fig. 1B) using primer set A, which amplifies the 5′-end of *runx2* (encoding isoforms II and III). Levels of *runx2* were higher in DU 145, PC-3 and bone-metastatic PC-3 vs. non-invasive PC-3 and LNCaP cells using this set of primers. No signal was detected in LNCaP sublines C4, C4-2, and C4-2B. MG-63, a human osteosarcoma cell line, was used as a positive control. This set of primers amplified two bands, of ~286 and ~320 bp. Sequence analysis confirmed the identity of the 286-bp (matches accession # AF010284) and 320-bp (matches accession # AF053952) amplicons as *runx2*. Runx2 mRNA was detected in all CaP cell lines tested using primer set B, which amplifies a 363-bp sequence present at the 3′-end of the gene in all three isoforms of *runx2* (Fig. 1C). Three bands of ~363, ~425, and ~440 bp were amplified. Sequence analysis confirmed that the 363-bp sequence matched the predicted amplicon of *runx2*. The 425-bp amplicon has a 60-bp insertion, reported in the literature as exon 8 insertion/deletion [25]. The larger band sequence was not determined. When examining samples of normal prostate and primary CaP from 11 patients, we observed *runx2* expression in all of these specimens using both primer sets.

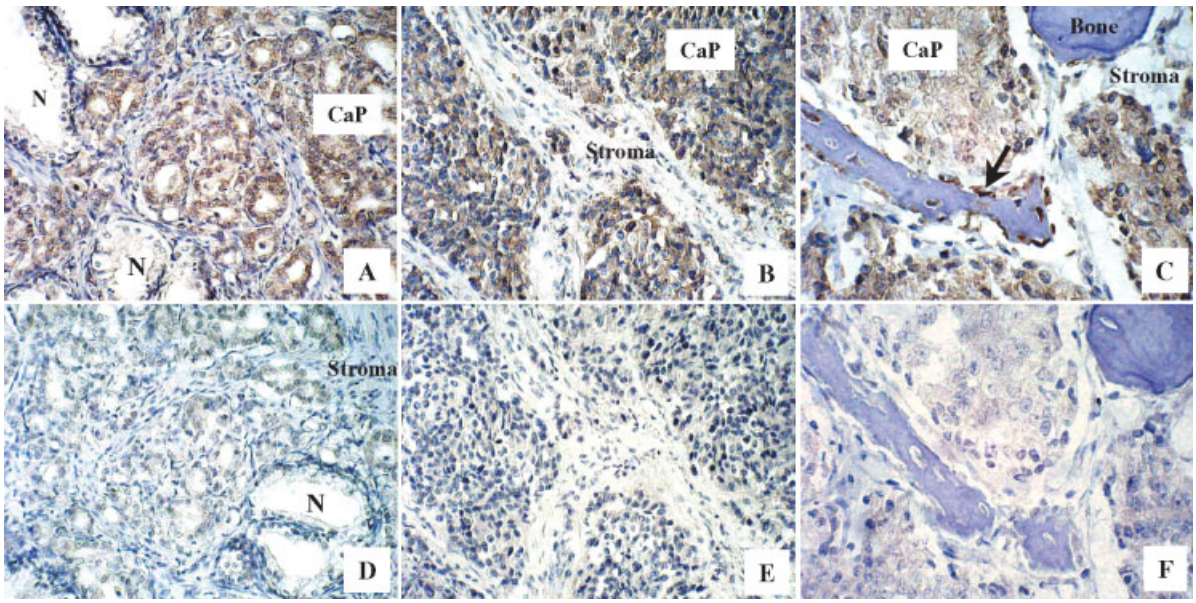

Fig. 2. Localization of Runx2 mRNA in CaP tissues by in situ hybridization. A representative sample of localized CaP shows Runx2 message in CaP cells (**panel A**) and luminal and basal cells of normal glands (N, panel A), while stromal cells (stroma) were negative. Runx2 message was also detected in CaP metastases, such as the lymph node metastasis in **panel B** and the osseous metastasis in **panel C**. Osteoblasts were highly positive for *runx2* (panel C, arrow). **Panels D–F**: They show the negative controls for each tissue. Magnification = 50×.

The presence of Runx2 mRNA was assessed in 6 samples of localized CaP, and 19 metastatic samples from 8 patients by ISH using riboprobes generated from primer set A. Runx2 mRNA was detected in epithelial cells of localized CaP in five of six samples (Fig. 2, panel A), and in basal and luminal cells of normal glands (2/3, Fig. 2, panel A, arrow) in the same sections. Runx2 mRNA was also detected in non-osseous metastases including adrenal (1/1), liver (1/1), and lymph node (5/5, Fig. 2, panel B) metastases. Osseous metastases were positive for Runx2 mRNA in 9/12 samples (Fig. 2, panel C). Osteoblasts present in bone metastases were intensely positive for Runx2 message (12/12). Staining with the sense probe was negative (panels D–F).

Runx2 protein

The presence of Runx2 protein was assessed in 10 samples of localized CaP and 6 osseous metastases by IHC. Runx2 protein was detected in CaP cells (10/10) (Fig. 3, panel A) and in basal and luminal cells of normal glands in the same sections (9/10). Osseous metastases were also positive for Runx2 protein expression (6/6, Fig. 3, panel C). Intense immunoreactivity was observed in osteoblasts in CaP bone metastases (6/6, Fig. 3, panel C, arrow). Staining for Runx2 was observed in both the nucleus and cytoplasm of CaP. The antibody used for IHC was tested by Western blotting for specificity, and detected protein of the expected molecular weight in both CaP cells and osteoblasts.

Expression of Runx2 protein in CaP cell lines was evaluated by Western blotting. Using rabbit anti-Osf-2 serum (against AAs 16-31 of isoform II, used for IHC studies), three proteins of 45, 55, and 60 kDa were detected in PC-3 and LNCaP nuclear extracts (Fig. 4A). Similar bands were observed in the nuclear extracts of two human osteosarcoma cell lines, MG-63 and Saos-2. Using anti-AML-3 polyclonal antibody (against AAs 333-348 of isoform II, found in all three isoforms), a 45-kDa band was detected in the nuclear extracts of PC-3, LNCaP, MG-63, and Saos-2 (Fig. 4B). The polyclonal antibody against AAs 294-363 (found in all three isoforms) of mouse Pebp2αA detected a protein of approximately 55 kDa in PC-3 and LNCaP nuclear extracts (Fig. 4C). In agreement with our RT-PCR results, basal levels of Runx2 protein were substantially higher in PC-3 than LNCaP, according to quantitative densitometry of the ECL signal (data not shown). The predicted sizes of the three isoforms are 56 (MRIPV), 57 (MASNS), and 65 (MLHSP) kDa. Given that we have observed messages of all three isoforms in CaP by RT-PCR, and isoforms II/III by ISH, the ~55-kDa band likely represents isoforms I and II, since their molecular weights are indistinguishable by Western blotting, and the 60-kDa band is probably isoform III. The 45-kDa protein may be the result of proteolytic cleavage or is

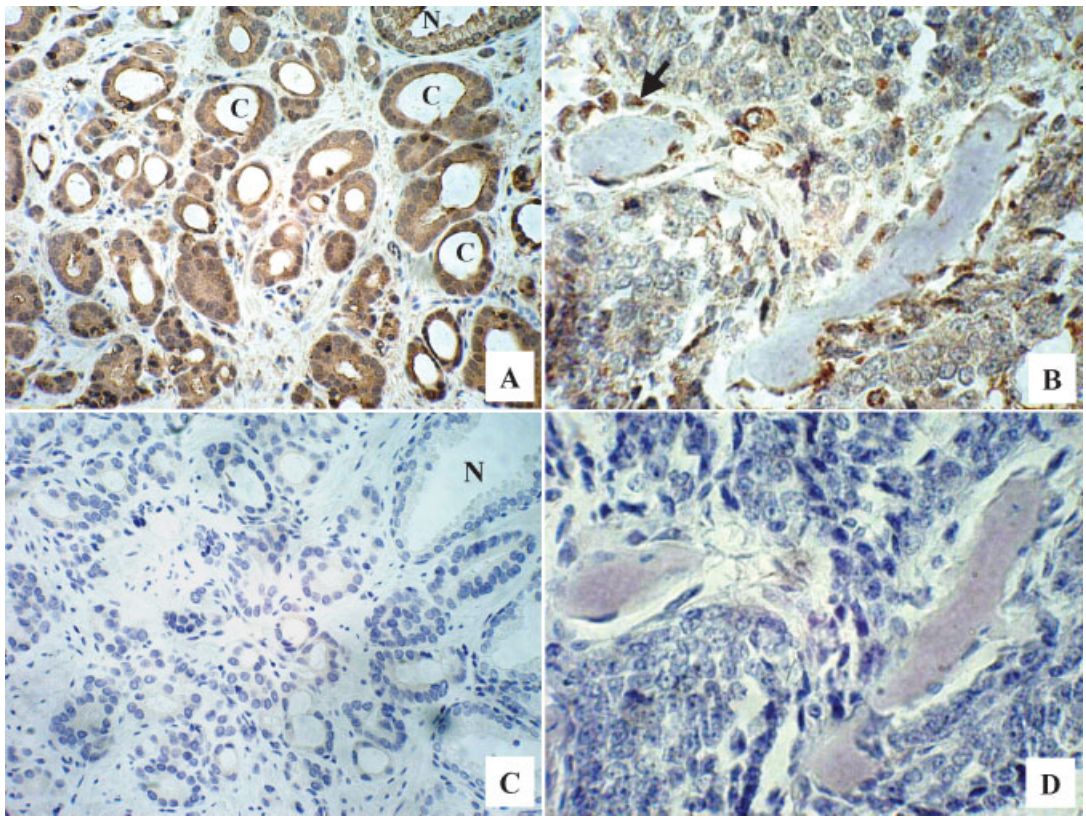

Fig. 3. Immunolocalization of Runx2 protein in CaP tissues. Runx2 protein was detected in CaP cells (C) and normal glands (N) in prostatic samples obtained from radical prostatectomis (**panel A**). CaP bone metastases were positive for Runx2 protein (**panel B**). Osteoblasts in bone metastases were also strongly positive (arrow, panel B). Staining for Runx2 was both nuclear and cytoplasmic. **Panels C–D**: They show the negative control sections. Magnification = 50×.

an unidentified variant and may warrant further investigation.

Runx2/OSE2 Interaction by Gel Supershifts

Protein/DNA complexes were observed in LNCaP and PC-3 nuclear extract samples using oligonucleotides containing either an AML binding consensus sequence [18] (Fig. 5A) or a human osteocalcin OSE2 sequence (Fig. 5B). A complex in the PC-3 samples was supershifted by the polyclonal antibodies anti-AML-3 and anti-Pebp2αA, demonstrating the presence of Runx2. In the LNCaP samples, only a faint supershift was observed with the AML binding sequence and the anti-Pebp2αA antibody.

BMP-2 Regulation of Runx2 Expression

BMP-2 has been reported to upregulate Runx2 protein levels in osteoblasts and their precursors [33–35]. We observed that BMP-2 treatment did not alter Runx2 protein levels in LNCaP or PC-3 (Fig. 6A), although it did upregulate the levels in the C_2C_{12} control cells (Fig. 6B). To determine whether BMP-2 treatment augments Runx2/DNA interactions, we examined the BMP-2 treated samples by EMSA, but did not observe an increase in protein/DNA interactions in LNCaP or PC-3 samples (data not shown). Although BMP-2 did not alter Runx2 levels in CaP cell lines, we did observe SMAD1 phosphorylation, indicating functional signaling of BMP-2 in CaP (data not shown).

DISCUSSION

The coordinate upregulation of a number of proteins related to bone metabolism in bone metastases of CaP indicates the presence of a controlling agent, such as a transcription factor, that may be responsible for some or all of these effects. We have shown that Runx2, a transcription factor associated with osteoblast differentiation, is expressed in CaP samples from various stages of disease progression. As pointed out above, this transcription factor is known to regulate transcription of a number of important bone-related factors. Therefore Runx2 is a likely candidate as the agent (or one of a group of agents) mediating the expression of these factors at the transcriptional level in CaP bone metastases.

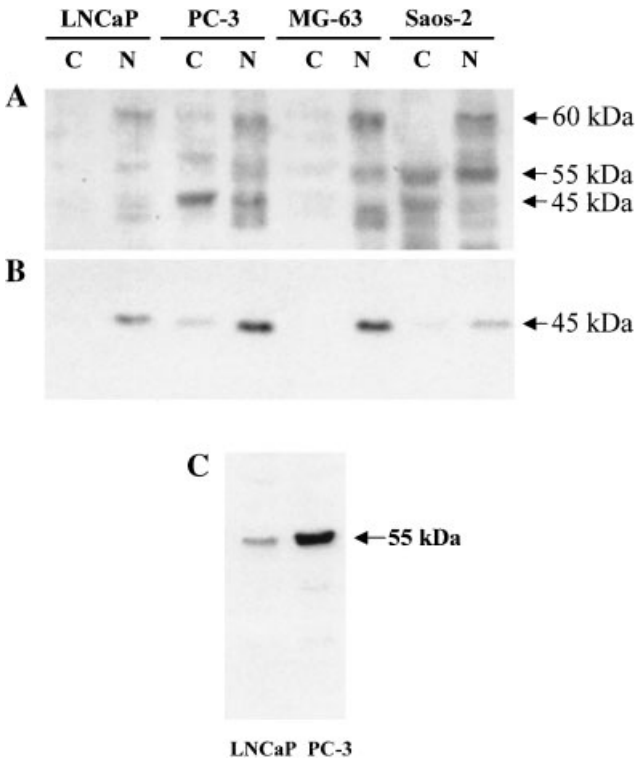

Fig. 4. Detection of Runx2 in CaP lines by Western blotting. **A**: Three proteins of 45, 55, and 60 kDa (arrows) were detected in nuclear extracts (N) of LNCaP, PC-3, and two osteoblastic cell lines, MG-63 and Saos-2 using rabbit sera raised against Osf-2 (same antibody used for IHC), while cytoplasmic (C) fractions contained bands of 45 and 55 kDa in the PC-3 and Saos-2 samples. **B**: The anti-AML-3 antibody detected a 45 kDa protein (arrow) in nuclear extract of all four cell lines. **C**: The anti-Pebp2αA antibody detected a 55 kDa (arrows) protein in LNCaP and PC-3 nuclear extracts.

We have detected Runx2 message and protein in CaP cell lines. Interestingly, we detected isoforms II and III, which are associated with osteoblastic cells in several CaP cell lines. In addition to detecting Runx2 isoforms in CaP, we also detected the alternate spliced form at exon 8, which affects transcriptional activity of the protein. Our results show that PC-3 cells express higher levels of Runx2 than LNCaP and its sublines. This phenomenon may be related to the origin of these cells; PC-3 cells originated from a CaP bone metastasis [36], and LNCaP cells were isolated from a lymph node metastasis [37]. In support of our hypothesis that Runx2 is involved in upregulation of bone-related proteins in CaP cells, we (unpublished data) and others have demonstrated that PC-3 cells express higher levels of bone related factors, such as OPG [38], PTHrP [39], endothelin-1 [40], and osteocalcin [28] than LNCaP and its sublines. Also, PC-3 cells grow more readily in the bone environment than LNCaP cells and its sublines. However, the absence of osteoblast specific isoforms II/III in LNCaP sublines, which grow more readily in the bone than parental LNCaP, is suggestive that these isoforms are not essential for these cells to survive in the bone environment. It should be noted that C4-2 and C4-2B cells express isoform I of *runx2*, which was also reported to stimulate expression of osteocalcin, osteopontin, and alkaline phospatase [27].

Although Runx2 protein was detected in both PC-3 and LNCaP nuclear extracts, formation of a specific Runx2/OSE2 (or AML) complex was readily observed in the PC-3 samples, while LNCaP only gave a faint supershift with the AML binding sequence and the anti-Pebp2αA antibody. Our supershift data are in agreement with results reported by Yeung et al. [28], who only observed supershifts with PC-3 nuclear extracts. The disparity between LNCaP and PC-3 Runx2 DNA binding abilities may be based on inherent properties of the cell lines or the assay itself. First, we observed that PC-3 has elevated levels of Runx2 compared to LNCaP based on RT-PCR and Western blots. Second, it is possible that a co-factor or activity required for formation of the protein/DNA complex in PC-3 may be absent or mutated in LNCaP. It has been reported that phosphorylation of Runx2 by MAP kinases increases its transcriptional regulation of osteocalcin [41]. We have observed high levels of basal p38 MAP kinase and ERK1/ERK2 activities in PC-3, while these activities are barely detectable in LNCaP (our unpublished data), which is in agreement with Putz et al. [42] who reported that LNCaP do not have basal levels of ERK2 activity in vitro. Therefore, signaling pathways may be required to activate Runx2 or another protein needed for protein: DNA interactions, which may be absent in LNCaP cells in vitro.

Our expression data in CaP cells are in accord with recent studies of Yeung et al. [28] and Lin et al. [29] demonstrating Runx2 expression in PC-3 and C4-2B, two CaP cell lines which express osteocalcin [1,28] and metastasize to bone. Despite these common features, these cell lines affect bone remodeling differently. PC-3 intra-tibial xenograft models display a lytic phenotype [43], while C4-2B xenografts demonstrate a mixed lesion characteristic of both resorption and bone formation [44]. Interestingly, we did not detect *runx2* isoforms II and III in C4-2B. Runx2 protein levels in C4-2B nuclear extracts were also lower than in LNCaP and PC-3 nuclear extracts by Western blotting (data not shown). Therefore, the relationship between the expression levels of Runx2 (including isoforms), propensity to metastasize to bone, and expression of osteocalcin levels is complex and may depend on various factors in the bone environment. One such factor reported to upregulate Runx2 levels in osteoblasts is BMP-2. We found that BMP-2 did not alter Runx2 protein levels in LNCaP and PC-3, but did upregulate the levels in a pre-myoblast (mesenchymal) cell line,

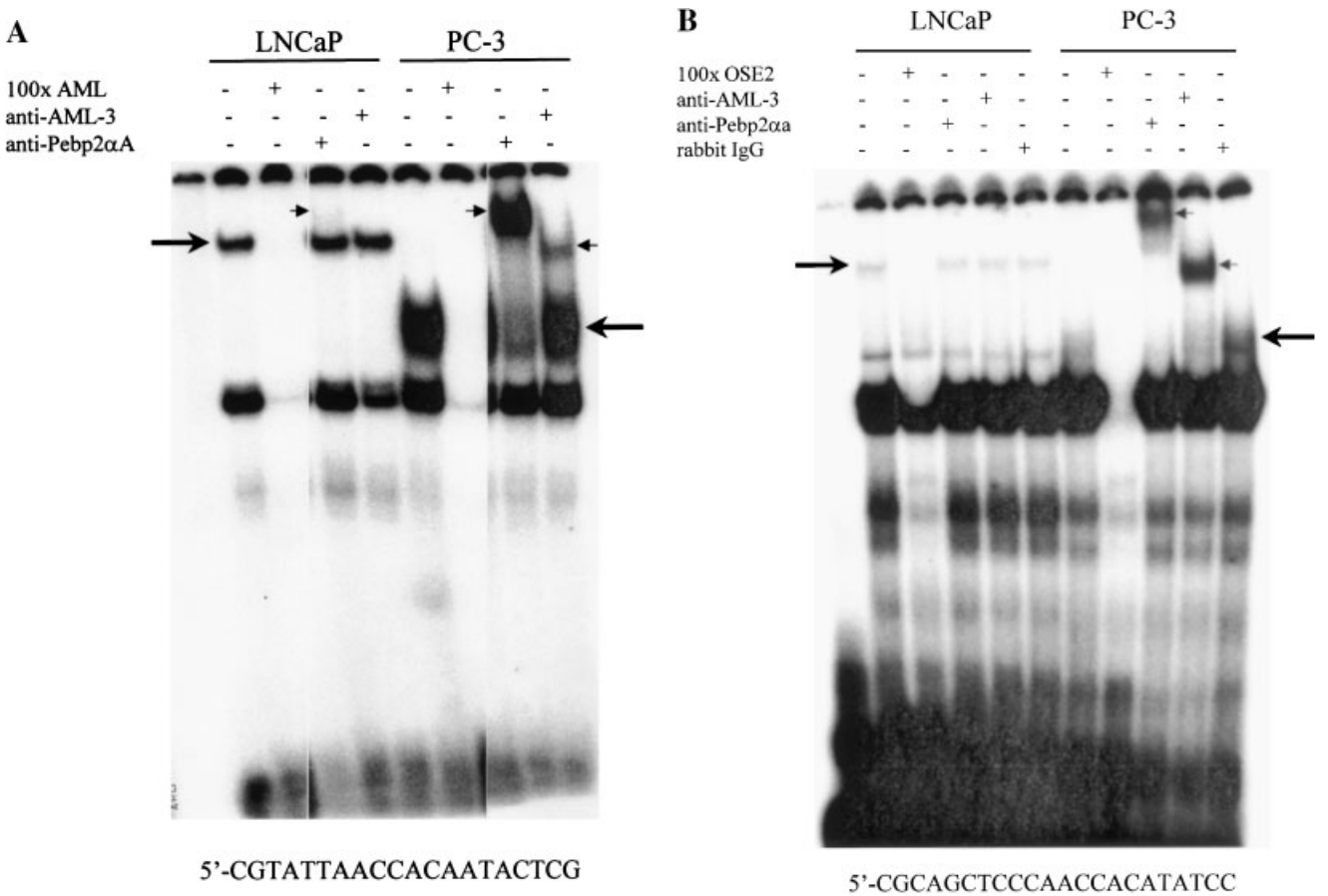

Fig. 5. Detection of Runx2: DNA binding complexes by EMSA. **A**: EMSA using an AML binding sequence. **Lane l** is labeled DNA alone. Protein: DNA complexes were observed for both LNCaP and PC-3 nuclear extracts (large arrows). Supershifts (small arrows) were observed when either anti-Pebp2αA or anti-AML-3 antibodies were added to the reactions, although LNCaP only gave a faint supershift with the anti-Peb2αA antibody. **B**: EMSA using an OSE2 in the human osteocalcin promoter. Similar Runx2: DNA complexes (large arrows) were detected as in (A). Supershifts (small arrows) were only observed for the PC-3 nuclear extracts using both antibodies. Lanes with l00 × AML or OSE2 were samples run in the presence of l00× unlabeled probe for competition studies.

C_2C_{12}. Lin et al. [29] reported upregulation of BMP-7 in C4-2B treated with promineralization media and hypothesized, but did not demonstrate a relationship between this upregulation and expression of Runx2.

In addition to our expression data in CaP cell lines, we also demonstrate that normal prostate and CaP from patient samples express Runx2 mRNA and protein. We detected isoforms II and III, which are associated with osteoblastic cells, in normal glands and primary CaP samples, and in CaP metastases to lymph nodes, liver, adrenal glands, and bone by in situ hybridization. We have also detected Runx2 protein by immunohistochemistry. Runx2 immunostaining in CaP cells was nuclear as well as cytoplasmic, implying that a portion of the protein is not imported into the nucleus. It is possible that factors needed for Runx2 translocation to the nucleus may be present at lower levels in the CaP cells. Our hypothesis is that expression of Runx2 in CaP may be the molecular switch that is associated with expression of various bone-specific factors in CaP, which in turn can influence bone remodeling and possibly play a role in the growth and survival of CaP. In addition, to detection of Runx2 in bone metastases, we have also detected Runx2 in normal prostate, primary CaP, and non-osseous CaP metastases. Others have reported expression of Runx2 in other tissues and cell types, such as testis [45], thymus [8], and T-cell lines [21,22]. The significance of expression and roles of Runx2 in non-osseous environment remains to be determined.

The "seed and soil" theory of Paget [46], and Koeneman and Chung's [1] argument that CaP acquires osteomimetic properties provide us with a theoretical framework for CaP adaptation to bone. However, the molecular mechanisms involved in this ability to acquire osteoblast-like properties have not been completely elucidated. Expression of Runx2 in CaP provides a plausible mechanism that could account for increased

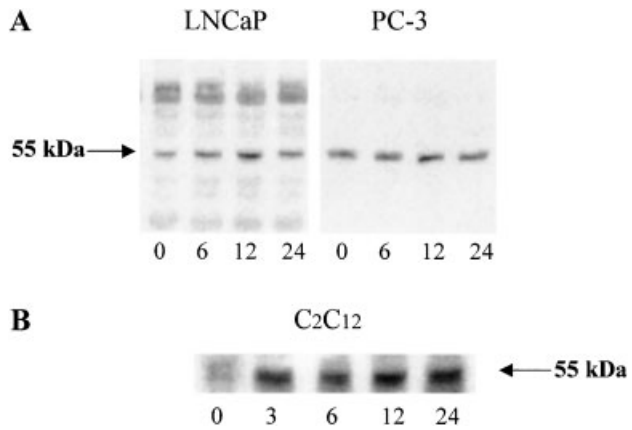

Fig. 6. BMP-2 regulation of Runx2 protein expression. **A**: Treatment of LNCaP and PC-3 with 200 ng/ml BMP-2 for 0, 6, 12, and 24 hr did not affect Runx2 (arrow) protein levels. The **left panel** is a 2-min exposure, while the **right panel** is a 5-sec exposure (from the same blot). **B**: Treatment of pre-myoblast C_2C_{12} with 200 ng/ml BMP-2 increased Runx2 protein levels at 3, 6, 12, and 24 hr of treatment. Runx2 protein levels were detected using the anti-Pebp2αA antibody.

expression of bone-related factors by CaP. The next step in understanding the role of Runx2 in CaP bone metastasis is elucidation of the factors and mechanisms regulating its expression in CaP, including the high basal levels observed in the PC-3 cell line.

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